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14. ABSTRACT Recent studies have implicated ectopic activation of the Wnt pathway in many human cancers, including breast cancer. β -catenin is a critical co-activator in this signaling pathway, and is regulated in a complex fashion by phosphorylation, degradation, and nuclear translocation. Glycogen synthase kinase-3 β (GSK3 β) phosphorylation of the N-terminal domain of β -catenin targets it for ubiquitination and proteosomal degradation. We hypothesized that expression of dominant negative (DN) GSK3 β in mammary glands would function in a dominant negative fashion by antagonizing the endogenous activity of GSK3 β and promoting breast cancer development. Consistent with this, we find that DN-GSK3 β stabilizes β -catenin expression, catalyzes its localization to the nucleus, and upregulates the downstream target gene, cyclin D1, <i>in vitro</i> . <i>In vivo</i> , transgenic mice overexpressing the DN-GSK3 β under the control of the MMTV-LTR develop mammary tumors with overexpression of β -catenin and cyclin D1. In addition, low dose of carcinogen treatment (DMBA) accelerates mammary tumor formation in DN-GSK3 β mice and exhibits a higher mortality rate than untreated transgenic mice. Thus, antagonism of GSK3 β activity is oncogenic in the mammary epithelium; mutation or pharmacologic downregulation of GSK3 β could promote mammary tumors. Moreover, carcinogen treatment accelerates tumorigenesis in mice that have a genetic predisposition to breast cancer.					
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Table of Contents

Introduction.....	4
Body.....	5-14
Key Research Accomplishments.....	15
Reportable Outcomes.....	15
Conclusions.....	16
References.....	17-18
Appendices.....	19-31

Introduction

With one million new cases in the world each year, breast cancer is the most common non-dermatologic malignancy in women and constitutes 18% of all female cancers. An increasing number of molecular markers have been detected in breast cancer patients in recent years, including group of genes that are associated with the Wnt signaling pathway.

β -catenin is the critical co-activator in this signaling pathway, and is regulated in a complex fashion by phosphorylation, nuclear translocation, and degradation. Glycogen synthase kinase-3 β (GSK3 β) phosphorylation of the N-terminal domain of β -catenin targets β -catenin for ubiquitination and proteosomal degradation.

While a role for the Wnt pathway is well-recognized in human colon cancer, where for example mutations of the APC and β -catenin genes are found in both sporadic and inherited cancers, little is known about the Wnt pathway in human mammary tumorigenesis. Overexpression of several Wnts has been reported in breast cancer (1-4) and amplification of the dishevelled downstream messenger has been seen in 50% of primary breast tumors (5). Elevation of β -catenin protein expression has been reported in 60% of human breast cancer tissues (6) and detection of β -catenin by immunohistochemistry has been associated with poor outcome (7). In this regard, GSK3 β has not been studied. We hypothesized that overexpression of a dominant negative form of GSK3 β (DN-GSK3 β) would also promote mammary tumorigenesis. A previous postdoctoral fellow in the lab cloned murine GSK3 β and created an enzymatically inactive form by mutating the ATP binding site. We then engineered transgenic mice overexpressing the DN-GSK3 β under the control of the MMTV-LTR. A cohort of 117 transgenic female mice derived from three independent DN-GSK3 β transgenic lines was observed for 2 years. Sixty-two percent of the mice developed mammary tumors at a median age of 22 months, in all three transgenic lines. Tumors from the MMTV-DN-GSK3 β transgenic model were usually classified as adenocarcinomas, with features of tumors of the Wnt pathway group, histopathologically different from the ErbB/Ras pathway breast tumors (8). The overall goal of this proposal is to unravel the molecular mechanism by which DN-GSK3 β promotes tumorigenesis and examine a possible cooperation between DN-GSK3 β with other Wnt pathways oncoproteins like protein kinase CK2 (CK2) to promote breast cancer.

Protein kinase CK2 is another key regulator of the Wnt signaling pathway in mammalian cells. Phosphorylation of β -catenin by CK2 occurs in the central armadillo repeat domain (9). This phosphorylation promotes Wnt signaling by antagonizing the effect of GSK3 β phosphorylation in the N-terminal domain. CK2 has been documented to be elevated in many human solid tumors, such as lung, prostate and head and neck, and in leukemias (10-15). In human breast cancer, strong nuclear staining of the CK2 α subunit in breast tumor tissue compared to a normal tissue was shown by immunohistochemistry (16).

Body

Task 1 proposes to study the changes in β -catenin expression and localization due to DN-GSK3 β . To work with the DN-GSK3 β construct *in vitro* and to be able to detect it in cells, I tagged it with HA in the N-terminus using specific primers. C57MG cells, a non-malignant murine breast epithelial cell line, were transiently transfected with increasing amounts of HA-tagged DN-GSK3 β and were analyzed for β -catenin expression. β -catenin protein levels were upregulated with increasing expression of the HA-DN-GSK3 β (Fig. 3A in appendix), consistent with increasing activation of the Wnt pathway.

To validate that the changes in β -catenin expression are due to ability of the HA-DN-GSK to stabilize β -catenin protein, the half-life of β -catenin was measured using cycloheximide (CHX) to block new protein synthesis. The half-life of β -catenin in the transfected cells was as long as 26.6 hrs (4 μ g plasmid) compared with only 2.2 hrs in untransfected cells (Fig. 3B in appendix).

To investigate if DN-GSK3 β can influence the translocation of β -catenin from the cytoplasm to the nucleus I applied two different methods. First, cytoplasmic and nuclear extracts were prepared from the transfected C57MG cells. In the control cells transfected with the empty vector alone the levels of β -catenin protein in the cytoplasm were higher than in the nucleus. In contrast, in the presence of increasing amounts of DN-GSK3 β , the levels of β -catenin in the nucleus were significantly higher than in the cytoplasm (Fig. 2A). Second, immunofluorescence was used to confirm nuclear translocation of the upregulated β -catenin. In the control C57MG cells (Fig. 2B, panels a and d in appendix), most of the β -catenin is located in the cytoplasm and in the plasma membrane. In contrast, the cells transfected with the HA-DN-GSK3 β plasmid exhibited strong nuclear staining (Fig. 2B, panels b and e in appendix).

The efficiency of the transient transfection experiments was very high (75%) using a nucleofection system (Amaxa) that was designed to deal with cells that are usually very hard to transfect. Hence, I did not have to establish stable transfectants.

In vivo, to study the association between DN-GSK3 β and β -catenin expression, I assayed the expression levels of β -catenin in mammary glands and tumor tissues from the DN-GSK3 β transgenic mice. β -catenin protein was upregulated in the tumor samples in 6 out of 7 transgenic mice (Fig. 7A, quantification in 7B in appendix).

Task II proposes to identify the downstream targets that are modulated by DN-GSK3 β . When β -catenin translocates into the nucleus during canonical Wnt signaling, it binds transcription factors of the TCF/LEF family and dramatically increases their activity, stimulating the expression of proto-oncogenes such as cyclin D1(10) (11). In a preliminary experiment, I used the TOPFLASH/FOPFLASH TCF/LEF luciferase reporter system (12) and showed that co-transfection of the reporter along with HA-DN-

mGSK3 β resulted in a ten-fold increase in luciferase activity compared with controls. To demonstrate this for an endogenous biologically relevant gene, I measured levels of cyclin D1 expression (a downstream Wnt target). To fulfill this task I used both C57MG cell lines that were transiently transfected with HA-DN-GSK3 β as well as tumor specimens that were derived from the DN-GSK3 β transgenic mice to study the mRNA and protein expression levels of cyclin D1.

In C57MG transfected cells that were subjected to qPCR, an increase in cyclin D1 mRNA of up to almost seven-fold was seen (Fig. 4C in appendix). Consistent with these results, the levels of cyclin D1 protein were higher in cells expressing HA-DN-GSK3 β compared to vector-transfected cells (Fig. 4A bottom panel in appendix).

Using normal mammary glands and tumor tissues from the DN-GSK3 β transgenic mice, cyclin D1 protein levels were upregulated in the tumor samples in 6 out of 7 transgenic mice (Fig. 7A in appendix). In addition, using qPCR, I compared expression of cyclin D1 in the mammary glands of wild type female FVB/N mice and DN-GSK3 β transgenics. The presence of the transgene resulted in a detectable increase in cyclin D1 mRNA (Fig. 7C, black bars in appendix) in the pre-malignant mammary gland as well as in malignant mammary gland.

Task III proposes to determine whether GSK3 β inhibitors promotes mammary tumorigenesis. To fulfill this task I used both siRNA and pharmacological inhibitors. C57MG cells were transiently transfected by nucleofection with SMARTpool siRNA, a pool of four specific siRNAs oligos for mGSK3 β , or siCONTROL. GSK3 β protein level was reduced significantly in cells transfected with the siRNAs for GSK3 β compared to siRNA control or untransfected cells (Fig. 5A in appendix). The efficiency of the transient transfection experiments was very high (75%) using the Amaxa nucleofection system, hence I did not use the siRNA plasmid based system.

There was an inverse correlation between the expression of GSK3 β and β -catenin, which was upregulated in cells transfected with GSK3 β siRNA. Alternatively, C57MG cells were treated with the GSK3 inhibitors SB216763, SB415385 or TDZD-8 at 20 μ M, 10 μ M and 5 μ M, respectively. As expected, the kinase inhibitors did not alter the levels of GSK3 β , but they did upregulate β -catenin expression as well as cyclin D1, consistent with inhibition of GSK activity and activation of Wnt signaling (Fig. 5B, C in appendix).

Thus, concluding the results from task I-III, I found that DN-GSK3 β stabilizes β -catenin expression, catalyzes its localization to the nucleus, and upregulates the downstream target gene, cyclin D1, *in vitro*. *In vivo*, transgenic mice overexpressing the DN-GSK3 β under the control of the MMTV-LTR develop mammary tumors with over-expression of β -catenin and cyclin D1. DN-GSK3 β construct acts similarly to specific GSK3 β siRNA and pharmacological inhibitors. Thus, antagonism of GSK3 β activity is oncogenic in the mammary epithelium; mutation or pharmacologic downregulation of

GSK3 β could promote mammary tumors. This work has been published in July 2005 in Cancer Research (attach in appendix).

Task V proposed to examine how DN-GSK3 β cooperates with other Wnt pathway oncoproteins known to promote breast cancer, such as protein kinase CK2 α .

In the mammary gland, transgenic CK2 α and DN-GSK3 β both promote mammary tumorigenesis and produce Wnt-type tumors, but the latency is extremely long. In this task, I crossed the CK2 α and the DN-GSK3 β transgenic mice, both controlled under the MMTV promoter, to investigate whether there is any collaboration between dysregulated CK2 α and DN-GSK3 β . Several pieces of data led me to choose CK2 α as a candidate for the establishment of bi-transgenic mice. First, our lab has shown that CK2 is a key regulator of the Wnt signaling pathway in mammalian cells. CK2 is upregulated by the presence of a Wnt-1 signal in mouse mammary epithelial cells and endogenous CK2 in mammalian cells associates with the Dvl proteins (17). Furthermore, β -catenin can also be found in this complex and is phosphorylated by CK2, which affects its stability and co-transcriptional activity. Second, our lab has also demonstrated that overexpression of the α catalytic subunit of CK2 in the mammary gland (MMTV-LTR-CK2 α) promotes mammary tumorigenesis in transgenic mice (18). 30% of the female transgenic mice develop mammary adenocarcinomas at a median of 23 months of age. Tumors from the MMTV-CK2 α transgenic model were classified histologically as belonging to the Wnt pathway tumor group, together with the DN-GSK3 β mice (8). The upregulation in β -catenin in most of the tumors arose from the CK2 α transgene is in congruent with the activation of β -catenin in DN-GSK3 β tumors. Third, CK2 α showed to synergize with other oncogenes such as myc and tal-1 and with loss of p53 in lymphomagenesis (19-21).

I did a sample size calculation based on the incidence of breast tumors in DN-GSK3 β transgenic of 10% at 18 months and assuming that bi-transgenic would have a 50% incidence of tumors at 18 months, and found that I needed 23 mice to have a 90% probability of finding a statistically significant difference at a p value of 0.05. To obtain sufficient numbers of bi-transgenic mice, I identified homozygous DN-GSK3 β mice from the DN-GSK3 β colony by out-crossing DN-GSK3 β with WT FVB mice. I genotyped all the litters using specific primers to the DN-GSK3 β transgene. Litters that were 100% positive for DN-GSK3 β indicated that their DN-GSK3 β parent was homozygous. I obtained three pairs of homozygous DN-GSK3 β mice and mated them with heterozygous CK2 α . All the litters derived from these pairs were positive for DN-GSK3 β and were genotyped for the CK2 α transgene using specific primers. Following this protocol, I have established 34 bi-transgenic mice to date that are continuously bred to induce both transgene expression through activation of the hormone-dependent MMTV-LTR. Mice are monitored weekly for the appearance of tumors. I am following the tumor

development of these mice to compare with CK2 α and DN-GSK3 β mono-transgenic mice. Acceleration of tumor development in the double transgenic mice will demonstrate collaboration in the carcinogenesis pathway.

Table 1 summarizes the results of the tumor incidence that we have so far and Fig. 1 shows representative histology. 9 out of 34 (26%) developed mammary tumors in a period of 29 to 96 weeks of age and another 6 were found dead. Two more mice had to be sacrificed due to severe illness. Nine tumors were harvested and sent for histological analysis; all were adenocarcinomas. The most common histological subtypes were adenosquamous (n=5), papillary adenocarcinoma (n=2) and one tumor was acinar. One mouse had a hyperplastic spleen with mild hepatitis but did not have a mammary tumor. So far, it appears that the latency of the tumors may be reduced, but it is still too soon to conclude whether there is collaboration between CK2 α and DN-GSK3 β .

Due to the fact that a long period of time and effort was put into task V, I have so far deferred task IV. The purpose of aim IV was to determine if DN-GSK3 β also cooperates with Wnt-1 to promote breast cancer. Instead, we sought another method to show acceleration of mammary tumors in DN-GSK3 β mice. I examined whether carcinogen treatment cooperates with DN-GSK3 β mice in developing mammary tumors.

Carcinogen-induced mammary tumors produced in rats by administration of the polycyclic aromatic hydrocarbon DMBA is a well-studied model system that has revealed molecular pathways of breast carcinogenesis (22, 23). This model is very relevant to human breast cancer because of the postulated role of environmental carcinogens in human mammary tumorigenesis. Recently, our lab in collaboration with others established an oral high and low dose regimen of DMBA in FVB mice. For the high dose protocol, five week old FVB female mice were given six weekly doses of 1 mg of DMBA administered by gavage and then bred continuously. Tumors arose at a median age of 20 weeks, 75% were mammary tumors with various histology analyses. Several oncogenic pathways including those involved in hydrocarbon signaling, Wnt signaling, and NF- κ B signaling were elevated in those mice. Specifically, elevated expression levels of several Wnt- signaling factors such as β -catenin, CK2 α and cyclin D1 were detected as well as elevated expression of aryl hydrocarbon receptor (AHR) and its cytochrome target CYP1B1 (24). For the low dose regimen, 20 five- weeks old FVB female, mice were given two doses of 0.5mg of DMBA one-week apart and then bred continuously. The incidence of tumor formation was very low during a period of 19 months.

On the basis of these data, and given the fact that DN-GSK3 β tumor latency in the mice is extremely low, I examined whether the low dose of carcinogen treatment (DMBA) accelerated mammary tumor formation in DN-GSK3 β mice. I treated 33 five week old DN-GSK3 β mice with the low dose DMBA protocol. 14 days later mice were bred to induce transgene expression through activation of the hormone-dependent MMTV-LTR. Mice were monitored weekly for the appearance of tumors. Figure 2 shows the survival curve for the mice. As seen, DN-GSK3 β mice treated with low dose DMBA had a higher mortality rate than untreated transgenic mice or FVB mice treated with low

dose DMBA. Table 2 summarizes the results from both experiments, including their histology. Out of 33 mice, 21 were found dead and 12 developed tumors. The median age for developing the tumors is 35 weeks (8 months). Tumors were harvested and sent for histology analysis. Of 12 for which we have histopathological reports, 5 tumors were adenocarcinomas and 2 were spindle cell tumors with epithelial to mesenchymal characteristics. The most common histological subtypes of adenocarcinoma were adenosquamous (n=4). One mouse developed papillary adenocarcinoma (n=1). Four mice had other tumors that are not in the mammary glands; two had lymphomas and two others had pulmonary adenomas. One mouse had splenomegaly with no mammary tumors. Thus, the presence of the DN-GSK3 β transgene potentiated the tumorigenic effect of low dose DMBA, producing mammary tumors and early mortality in the treated transgenic mice.

On the basis of these results we were interested to further elucidate the putative cooperation between AhR and DN-GSK3 β *in vitro*. We assayed the expression levels of AhR in histologically normal mammary glands and tumor tissues from the DN-GSK3 β transgenic mice. AhR protein levels were upregulated in the tumor samples in 5 out of 6 transgenic mice (Fig. 3A,B). When AhR expression levels in those tumors were compared to mammary glands from normal FVB mice, the differences were more significant (Fig. 3C). In addition, we examine the AhR levels in Wnt induced cell lines and in cells that were treated with pharmacological inhibitors for GSK3 β . Both experiments result in upregulation of AhR levels (Fig4).

Table 1: Histopathology of tumors derived from bi-transgenic mice MMTV-CK2 α -DN-GSK3 β

Mouse number	Age (weeks)	Histology report/other
8133	96.57	DIFFUSE LOBULAR HYPERPLASIA, ADENOSQUAMOUS CARCINOMAS (2), MAMMARY GLANDS
8135		
8137		
8142		
8143		found dead
8144		
8148	48.29	Lobuloalveolar hyperplasia MG tumor. Highly vascular. Erbb2 type solid tumor
8145		
257	93.29	sac'd due to bad illness
8772		
8774	70.29	ACINAR CARCINOMA AND LOBULAR HYPERPLASIA, MAMMARY GLAND
8773	88.14	DIFFUSE LOBULAR HYPERPLASIA, ADENOSQUAMOUS CARCINOMAS (2), MAMMARY GLANDS
256	55.29	sac'd due to bad dermatitis
8149	61.86	found dead
8111	64.86	found dead
8112	69.57	MULTIPLE MICROPAPILLARY ADENOCARCINOMAS AND LOBULAR HYPERPLASIA, MAMMARY GLAND.
1418		
1419		
1752		
1739	62.00	MULTIPLE MICROPAPILLARY ADENOCARCINOMAS AND LOBULAR HYPERPLASIA, MAMMARY GLAND.
1499	66.14	ADENOSQUAMOUS CARCINOMA AND LOBULAR HYPERPLASIA, MAMMARY GLAND.
1276		
1761		
1760	29.14	Hyperplastic spleen with mild hepatitis.
1759		
1309		
9094		found dead no tumors
9096		
624	29.00	found dead
643		
1399		
8126	39.43	found dead
8127	40.14	found dead
1133	38.86	ADENOSQUAMOUS CARCINOMA AND HYPERPLASIA, MAMMARY GLAND. SPLENIC HYPERPLASIA.

Figure 1

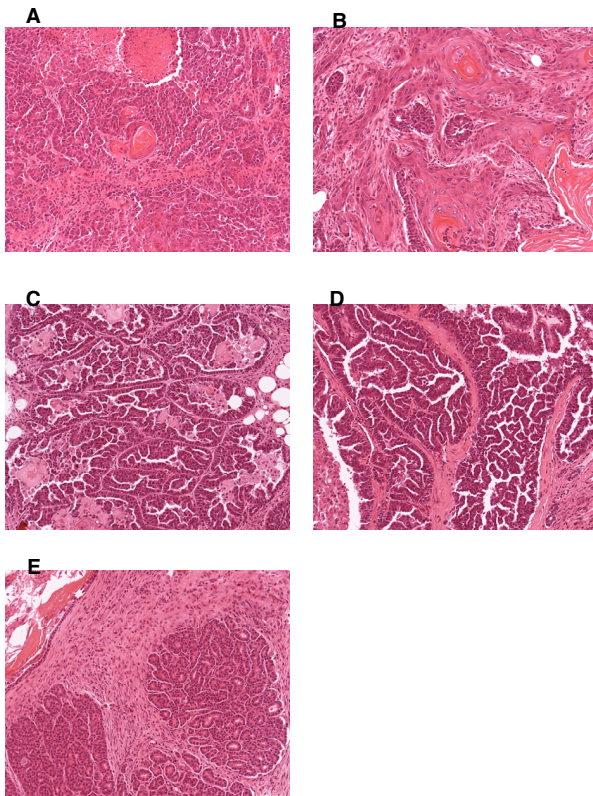


Fig 1. Varying histology of MMTV-DN-GSK3 β transgenic mouse mammary tumors, consistent with Wnt-like phenotype. A,B adenosquamous carcinomas. C,D micropapillary adenocarcinomas, . E. Acinar carcinoma.

Figure 2

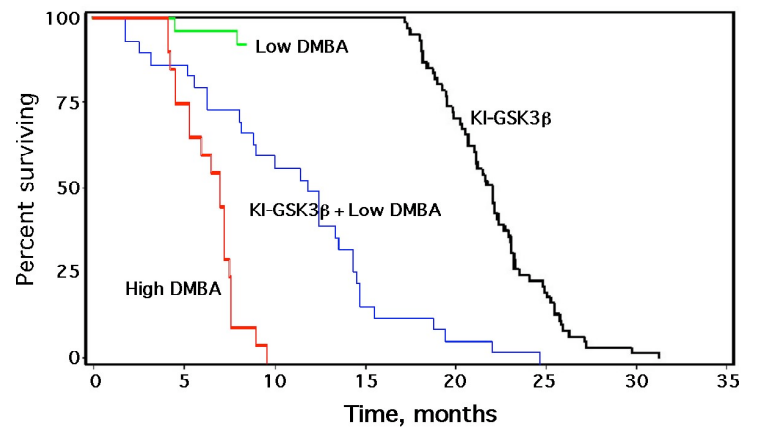


Fig2: Kaplan-Meier survival plot of DN-GSK3 β transgenic mice treated with low dose DMBA compared to untreated mice or FVB mice treated with high or low dose DMBA.

Mouse number	Age (weeks)	Histology/other
8302	6.43	found dead
8305	6.43	found dead
1775	9.43	found dead (24 days after second dose)
8348	12.14	found dead
8342	20.43	Myeloid immaturity, spleen and spindle cell tumors
8364	21.86	found dead
8365	24.86	found dead
8375	24.86	found dead
1417	32.12	Adenosquamous. Leukemic infiltrate and abscess,MG.
8369	32.43	found dead
1772	35.14	Spindle cell tumor (suggesting EMT) and enlarged spleen with increased myeloid immaturity
1770	35.71	Lymphocytic lymphoma involving multiple organs
1774	40	found dead
1771	45.71	Bronchial adenoma,lung. Squamous papilloma skin
1758	47.43	found dead
8361	49.86	found dead
8368	49.86	found dead
8314	49.86	found dead
8373	53.57	found dead
8350	54.29	Pulmonary adenoma, focal necrosis liver.Peritonitis with peri-uterine abscess,Pancreatitis and necrotic spleen
8366	57.57	Adenosquamous carcinoma (KA) MG, Congested spleen
8315	57.57	found dead
1763	58.29	dead
8360	59.14	papillary adenocarcinoma with pulmonary metastasis.Engorged spleen
1740	59.14	retroorbital abscesses,eye. Splenomegaly with increased myeloid immaturity. Liver,MG and lung are normal
8362	62.29	Malignant lymphoma, involving multiple organs
8344	75.57	sent to path
8355	78.29	found dead
8371	88.86	dead
1762	99.57	Complex secretory adenosquamous carcinoma,MG.Bronchial adenoma,Lung.
8345		found dead
8374		found dead
8304		found dead

Figure 3

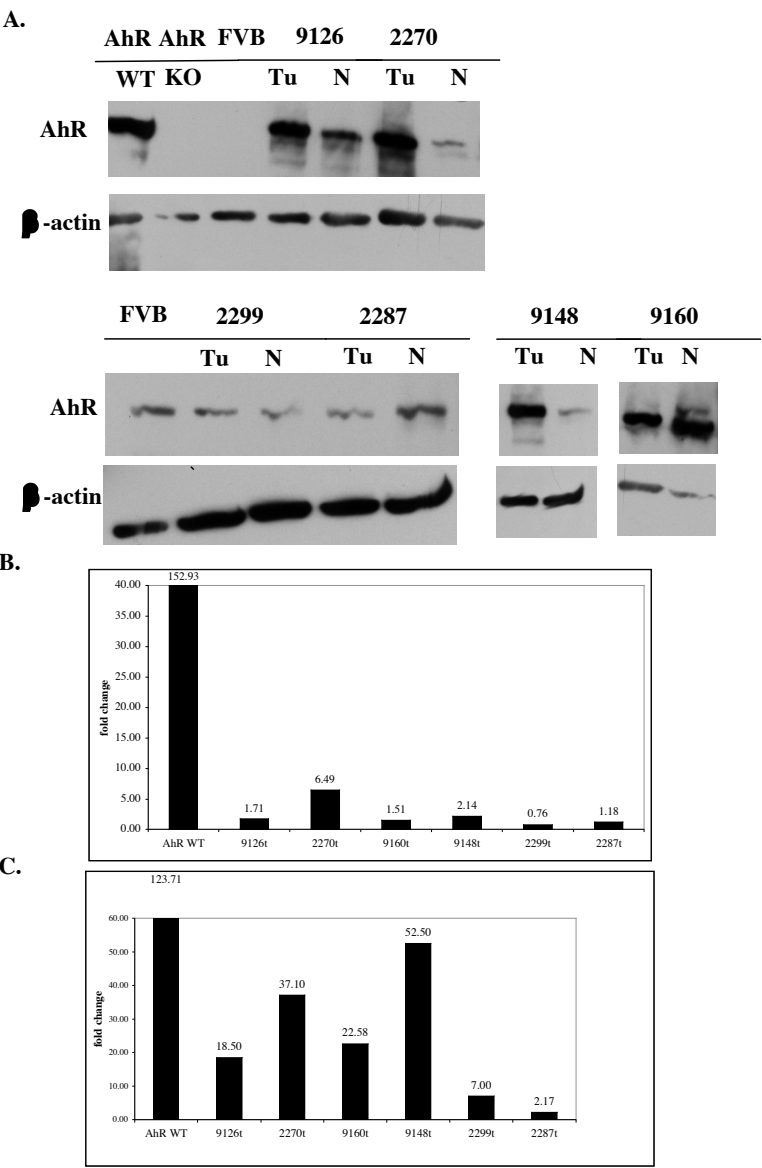


Fig 3. Expression of AhR protein in transgenic MMTV-DN-GSK3 β breast tumors.

A, Protein extracted from paired histologically normal mammary glands (N) and mammary tumors (Tu) from MMTV-DN-GSK3 β transgenic mice were subjected to immunoblotting for AhR, and to β -actin as a loading control. AhR WT and AhR KO are positive and negative control for AhR expression, respectively. The ratio of AhR protein expression in tumor vs. histologically normal glands from the same mice is plotted in panel B. The ratio of AhR protein expression in tumor vs. average expression of two normal glands from FVB mice is plotted in panel C.

Figure 4

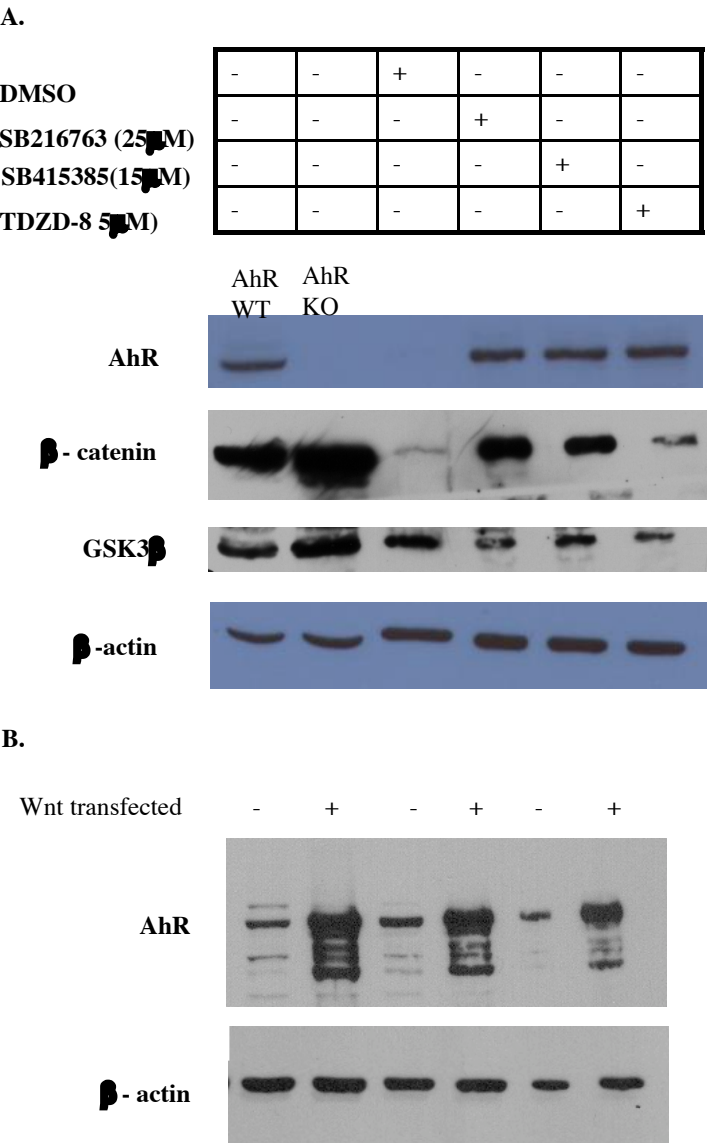


Fig 4. Inhibition of mGSK3 β using pharmacologic inhibitors or expression of Wnt ligand upregulates AhR expression.

A, pharmacologic inhibitors of GSK3 β upregulate AhR expression. C57MG cells were treated with DMSO vehicle control (0), SB216763 (25 μ M), SB415385 (15 μ M) or TDZD-8 (5 μ M). Protein extracted from the cells was subjected to immunoblotting for AhR, GSK3 β and β -catenin; β -actin was used as a loading control. B, Stable expression of Wnt1 upregulates AhR levels. Protein extracts from C57MG cells or C57MG cells stably

Key research accomplishments

- Optimized an Amaxa protocol for transfecting C57MG cell lines, to overcome low yields of transfection with conventional reagents.
- Optimized a siRNA protocol for transfecting C57MG cell lines.
- Calibrating different pharmacological inhibitors of GSK3 β to use in a suitable concentration on C57MG cell lines.
- Establishment of bi-transgenic mice MMTV-CK2 α -DN-GSK3 β that develop mammary tumors.
- Helping to establish an oral high and low dose regimen of DMBA in FVB mice.
- Finding that carcinogen treatment accelerates tumorigenesis and high mortality rate in mice that have a genetic predisposition to breast cancer.

Reportable Outcomes

- Publications
Marganit Farago, Isabel Dominguez, Esther Landesman-Bollag, Xin Xu, Andrea Rosner, Robert D. Cardiff, and David C. Seldin. "Kinase Inactive GSK3 β Promotes Wnt Signaling and Mammary Tumorigenesis". Cancer Res 2005; 65(13):5792-5801.
- Abstracts and presentations

Poster-Marganit Farago, Isabel Dominguez, Esther Landesman, Xin Xu, and David C. Seldin Farago, June 2005. "Kinase inactive GSK3 β promotes Wnt signaling and mammary tumorigenesis" Era of Hope 2005 DoD Breast Cancer Research Program Meeting, Philadelphia, Pennsylvania.

Oral presentation- Marganit Farago, Isabel Dominguez, Esther Landesman-Bollag, and David C. Seldin. 2004 "Kinase Inactive GSK3 β Promotes Wnt Signaling and Mammary Tumorigenesis" BRCA (Boston Cancer Research Association) meeting, Boston medical school, Boston, USA.

Poster-Farago Marganit, Xu Xin, Patel Sandip, Rosner Andrea, Cardiff Robert, Dominguez Isabel and Seldin David. 2004. "The role of DN-GSK3 β in mammary tumorigenesis" 10TH Annual student achievement day, Boston medical school, Boston, USA

Poster -Farago M, Dominguez I, Landesman-Bollag E, Xu X, Patel S, Rosner A, Cardiff R and Seldin D. 2004. "Multiple approaches to inhibition of GSK3 and activation of wnt signaling" 92ND EVANS research day, Boston medical school, Boston, USA

Conclusions

The results obtain so far indicate that

- DN -GSK3 β stabilizes β -catenin expression, catalyzes its localization to the nucleus, and upregulates the downstream target gene, cyclin D1, *in vitro*.
 - *In vivo*, transgenic mice overexpressing the DN-GSK3 β under the control of the MMTV-LTR develop mammary tumors with over-expression of β -catenin and cyclin D1.
 - The mutant HA-DN-GSK3 β acts similarly to specific GSK3 β siRNA and pharmacological inhibitors.
 - Carcinogen-induced mammary tumors produced in FVB mice by administration of the polycyclic aromatic hydrocarbon DMBA.
 - Low dose of carcinogen treatment (DMBA) accelerates mammary tumor formation in DN-GSK3 β mice (median age of 8 months compare to 22 months in untreated mice).
 - DN-GSK3 β mice treated with low dose DMBA has a higher mortality rate than untreated transgenic mice or FVB mice treated with low dose DMBA.
- Thus, antagonism of GSK3 β activity is oncogenic in the mammary epithelium; mutation or pharmacologic downregulation of GSK3 β could promote mammary tumors.

In conclusion, cancer is a complex multistep process proceeding from cellular transformation and development of a primary localized tumor to advanced metastatic disease. Understanding each step in this process is important and contributes to cancer therapy. Environmental pollutants as well as different molecular pathways are associated with tumorigenesis and metastasis. Many human cancers carry mutations in at least one component of the Wnt-signaling pathway. Thus, targeting the Wnt-signaling pathway represents one potential route for cancer treatment. While mutations in APC and β -catenin have been reported in colon cancer and mutations in axin were found in hepatocellular carcinoma, no mutations in these components have been found in breast cancer. The accumulation of β -catenin protein in breast tumors implies that additional components of the Wnt-signaling pathway can be mutated and contribute to tumor formation in breast cancer.

The proposal has focused on GSK3 β as a potential tumor suppressor and investigated whether its mis-expression is capable of perturbing the Wnt pathway and promoting breast cancer development, using *in vitro* and *in vivo* models. The results suggest that GSK3 β has the capability to be a tumor suppressor, and mutations or dysregulation should be sought in human sbreast cancer specimens. Moreover, there is collaboration between GSK3 β dysregulation and environmental carcinogen exposure in breast cancer formation.

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Appendices

Abstracts for meetings:

Era of Hope 2005 DoD Breast Cancer Research Program Meeting, Philadelphia, Pennsylvania.

KINASE INACTIVE GSK3 β PROMOTES WNT SIGNALING AND MAMMARY TUMORIGENESIS

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Recent studies have implicated ectopic activation of the wnt pathway in many human cancers, including breast cancer. β -catenin is the critical transcriptional co-activator in this signaling pathway, and is regulated in a complex fashion by phosphorylation, degradation, and nuclear translocation. Glycogen synthase kinase-3 β (GSK3 β) phosphorylation of the N-terminal domain of β -catenin targets it for ubiquitination and proteosomal degradation.

We hypothesized that expression of kinase inactive (KI) GSK3 β in mammary glands would function in a dominant negative fashion by antagonizing the endogenous activity of GSK3 β , perturbing the Wnt pathway and promoting breast cancer development.

We have established transgenic mice overexpressing KI-GSK3 β under the control of the mouse mammary tumor virus (MMTV) LTR. More than 60% of the female KI-GSK3 β mice have developed mammary tumors. We used tumor specimens from these KI-GSK3 β transgenic mice, and breast cancer cell lines transfected with a KI-GSK3 β expression construct to identify downstream targets that are modulated by KI-GSK3 β *in vivo*. Wnt pathway activation was assessed by measurement of β -catenin stabilization and nuclear translocation, and by expression of cyclin D1 expression by real time PCR and immunoblotting. We compared our results to those obtained with other methods of antagonizing GSK3 β : siRNA to reduce GSK3 β mRNA; or pharmacologic inhibitors of GSK3 β activity.

We found that KI-GSK3 β stabilizes β -catenin expression, catalyzes its localization to the nucleus, and upregulates the downstream target gene, cyclin D1, *in vitro*. Transgenic mice overexpressing the KI-GSK3 β under the control of the MMTV-LTR develop mammary tumors with over-expression of β -catenin and cyclin D1 *in vivo*.

There is growing evidence that human breast tumors are associated with dysregulation of many genes, including genes belonging to the Wnt signaling pathway. Defining mechanisms by which modulation of GSK3 β activity affects downstream pathways and contribute to breast cancer transformation will shed light on putative

mechanisms of breast carcinogenesis in women. These results are particularly important in light of the development of inhibitors of GSK3 β for treatment of diabetes mellitus.

The U.S. Army Medical research and Materiel command under W8IXWH-04-1-0375 supported this work.

BRCA (Boston Cancer Research Association) meeting, Boston medical school, Boston, USA.

KINASE INACTIVE GSK3 β PROMOTES WNT SIGNALING AND MAMMARY TUMORIGENESIS

Marganit Farago, Isabel Dominguez, Esther Landesman , Xin Xu, and David C. Seldin

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Recent studies have implicated ectopic activation of the wnt pathway in many human cancers, including breast cancer. b-catenin is the critical co-activator in this signaling pathway, and is regulated in a complex fashion by phosphorylation, degradation, and nuclear translocation. Glycogen synthase kinase-3b (GSK3b) phosphorylation of the N-terminal domain of b-catenin targets it for ubiquitination and proteosomal degradation.

We hypothesized that expression of kinase inactive (KI) GSK3b in mammary glands would function in a dominant negative fashion by antagonizing the endogenous activity of GSK3b and promoting breast cancer development.

Consistent with this, we find that KI-GSK3 β stabilizes β -catenin expression, catalyzes its localization to the nucleus, and upregulates the downstream target gene, cyclin D1, *in vitro*. *In vivo*, transgenic mice overexpressing the KI-GSK3 β under the control of the MMTV-LTR develop mammary tumors with over-expression of β -catenin and cyclin D1. The same results were obtained by using alternative methods to antagonized GSK3 β ; siRNA to reduce GSK3 β mRNA and pharmacologic inhibitors of GSK3 β activity.

Thus, antagonism of GSK3b activity is oncogenic in the mammary epithelium; mutation or pharmacologic downregulation of GSK3b could promote mammary tumors.

The U.S. Army Medical research and Materiel command under W8IXWH-04-1-0375 supported this work.

10TH Annual student achievement day, Boston medical school, Boston, USA
THE ROLE OF DN-GSK3 β IN MAMMARY TUMORIGENESIS

M.Farago, X.Xu, S. Patel, Rosner A, R. Cardiff, I. Dominguez and D. Seldin

Recent studies have implicated ectopic activation of the Wnt pathway in many human cancers, including breast cancer. β -catenin is the critical co-activator in this signaling pathway, and is regulated in a complex fashion by phosphorylation, degradation, and nuclear translocation. Glycogen synthase kinase-3 β (GSK3 β) phosphorylation of the N-terminal domain of β -catenin targets it for ubiquitination and proteosomal degradation. We hypothesized that expression of kinase inactive (KI) GSK3 β in mammary glands would work as a dominant negative, antagonizing the endogenous activity of GSK3 β upregulating Wnt signaling, and promoting breast cancer development. Consistent with this, we find that KI-GSK3 β stabilizes β -catenin expression and catalyzes its localization to the nucleus, and upregulates the downstream target gene, cyclin D1, *in vitro*. *In vivo*, transgenic mice overexpressing the KI-GSK3 β under the control of the MMTV-LTR develop mammary tumors with over-expression of β -catenin and cyclin D1. Thus, we conclude that GSK3 β is a tumor suppressor gene in the mammary epithelium. Mutation or pharmacologic downregulation of GSK3 β could promote mammary tumors.

The U.S. Army Medical research and Materiel command under W8IXWH-04-1-0375 supported this work.

92ND EVANS research day, Boston medical school, Boston, USA

MULTIPLE APPROACHES TO INHIBITION OF GSK3 β AND ACTIVATION OF WNT SIGNALING

Farago M, Dominguez I, Landesman-Bollag, E, Xu X, Patel S, Rosner A, Cardiff R, and Seldin D.

Objective: We have previously shown that expression of a kinase-inactive (KI) form of glycogen synthase kinase 3 β (GSK3 β) in cells and in transgenic mice promotes Wnt pathway activation and mammary tumorigenesis. Here we now examine in more detail the molecular consequences of this, and compare alternative approaches to inhibition of GSK activity. **Methods:** GSK3 β activity was downregulated using the KI mutant, siRNA, and new pharmacologic agents. Wnt pathway activation was assessed by measurement of β -catenin stabilization and nuclear translocation, and by expression of cyclin D1 expression by real time PCR and immunoblotting. **Results:** All of these reagents similarly stabilize β -catenin protein expression and promote nuclear translocation, with upregulation of the downstream target gene, cyclin D1. **Conclusions:** GSK3 β has the capability to be a tumor suppressor,

The U.S. Army Medical research and Materiel command under W8IXWH-04-1-0375 supported this work.

Kinase-Inactive Glycogen Synthase Kinase 3 β Promotes Wnt Signaling and Mammary Tumorigenesis

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Abstract

Recent studies have implicated ectopic activation of the Wnt pathway in many human cancers, including breast cancer. β -catenin is a critical coactivator in this signaling pathway and is regulated in a complex fashion by phosphorylation, degradation, and nuclear translocation. Glycogen synthase kinase 3 β (GSK3 β) phosphorylation of the NH₂-terminal domain of β -catenin targets it for ubiquitination and proteosomal degradation. We hypothesized that expression of kinase-inactive GSK3 β (KI-GSK3 β) in mammary glands would function in a dominant-negative fashion by antagonizing the endogenous activity of GSK3 β and promoting breast cancer development. Consistent with this, we find that KI-GSK3 β stabilizes β -catenin expression, catalyzes its localization to the nucleus, and up-regulates the downstream target gene, *cyclin D1*, *in vitro*. *In vivo*, transgenic mice overexpressing the KI-GSK3 β under the control of the mouse mammary tumor virus-long terminal repeat develop mammary tumors with overexpression of β -catenin and *cyclin D1*. Thus, antagonism of GSK3 β activity is oncogenic in the mammary epithelium; mutation or pharmacologic down-regulation of GSK3 β could promote mammary tumors. (Cancer Res 2005; 65(13): 5792-801)

Introduction

Glycogen synthase kinase 3 β (GSK3 β) is a serine/threonine kinase that was originally found to have a pivotal role in glycogen metabolism and insulin-mediated signaling but is now recognized to function in multiple biological pathways. More than 40 proteins have been reported to be phosphorylated by GSK3 β , including over a dozen transcription factors (1). Recently, attention has focused on the developmental role of GSK3 β . During fly development, the GSK3 homologue, zeste-white 3, is a negative regulator of wingless (wg) signaling, the agonist responsible for normal wing development. The vertebrate homologues of wg, the Wnts, are responsible for embryonic patterning beginning with the establishment of the embryonic axes (2). In *Xenopus* development, the dorsoventral axis is established by dorsal accumulation of β -catenin, a critical coactivator in the Wnt signaling pathway (3). Positive elements of the Wnt canonical pathway (e.g., β -catenin) produce ectopic axes when injected ventrally, whereas inhibitors or negative regulators of the pathway antagonize dorsalization. Rat, human, and *Xenopus*

GSK3 β block dorsalization, whereas inactive mutants of GSK3 β act as dominant negatives of the normal enzyme function, inducing axis duplication when injected ventrally in the embryo (4–6).

Biochemical studies have elucidated the role of GSK3 β in the canonical Wnt signaling pathway. In the absence of Wnt signals, free cytoplasmic β -catenin is incorporated into a cytoplasmic complex that includes Axin, GSK3 β , and adenomatous polyposis coli (APC). This enables casein kinase I to phosphorylate β -catenin, creating a consensus site on β -catenin for phosphorylation by GSK3 β . The phosphorylated β -catenin is then targeted for ubiquitin-mediated proteasomal degradation (7). This process is opposed by casein kinase 2 (CK2), which phosphorylates β -catenin in the armadillo repeat region, stabilizing it and promoting Wnt signaling and dorsal axis formation (8–10). Wnt signaling via Dishevelled (Dsh) inactivates GSK3 β and prevents it from phosphorylating β -catenin, reducing its affinity for axin and APC and stabilizing it in the cytoplasm. As β -catenin accumulates, it translocates into the nucleus, where it binds to T-cell factor (TCF) and lymphoid-enhancing factor (LEF) transcription factors and dramatically increases their transcriptional activity. Genes up-regulated by TCF/LEF include embryologic genes, such as *siamois* and *engrailed* (11), and adult proto-oncogenes, such as *c-myc* and *cyclin D1* (12–14).

In the mammary gland, canonical Wnt signaling seems to play a role in both development and cancer. Wnt 6 and Wnt 10b are expressed on the surface of the ectoderm in mammary placodes and buds beginning on embryonic day 11.25 and are essential for initiation of mammary morphogenesis (15, 16). LEF-1 is expressed in the mammary gland beginning at embryonic day 11.5, and in LEF-1 deficient mice, the gland fails to develop (17). Δ N89- β -catenin, a mutant of β -catenin that lacks the NH₂-terminal GSK3 β phosphorylation sites and is thereby stabilized, promotes precocious alveolar development during puberty (18). Negative regulators of Wnt signaling block mammary gland development: ectopically expressed Dickkopf, a Wnt pathway inhibitor, blocks early mammary gland development (19); other negative regulators of β -catenin inhibit alveolar development in pregnancy (20).

Although a role for the Wnt pathway is well recognized in colon cancer, where, for example, mutations of the *APC* and *β -catenin* genes are found in both sporadic and inherited cancers, little is known about the Wnt pathway in human mammary tumorigenesis. Overexpression of several Wnts has been reported in breast cancer (21–24) and amplification of the Dsh downstream messenger has been seen in 50% of primary breast tumors (25). Up-regulation of β -catenin mRNA levels has been detected by microarray analysis in human breast cancer (26) and elevation of β -catenin protein expression has been reported in 60% of human breast cancer tissues (27). Detection of β -catenin by immunohistochemistry has been associated with poor outcome (28, 29). These events have been modeled in mice, as mammary gland tumors develop in transgenic

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mice overexpressing genes in the Wnt signaling pathway, including Wnt 1 (30), Wnt 10b (31), Δ N89- β -catenin (18), and cyclin D1 (32). In contrast, in transgenic mice that overexpress Axin, the expression of cyclin D1 is attenuated and increased apoptosis occurs in the mammary epithelia (33). Overexpression of the regulator CK2 α also promotes mammary tumorigenesis (34). In this regard, GSK3 β has not been studied. In this article, we use a novel mouse model to explore the effect of Wnt pathway deregulation on the development of breast cancer, with emphasis on GSK3 β as a pivotal kinase regulator in this pathway. Because inactive mutants of GSK3 β can activate canonical Wnt signaling, we hypothesized that overexpression of kinase-inactive GSK3 β (KI-GSK3 β) in mammary glands would work as a dominant negative, antagonizing the endogenous activity of GSK3 β . Consistent with this, we find that kinase-inactive murine GSK3 β (KI-mGSK3 β) stabilizes β -catenin expression and catalyzes its localization to the nucleus and up-regulates the downstream target gene, *cyclin D1*, *in vitro*. *In vivo*, transgenic mice overexpressing the mutant form of GSK3 β under the control of the mouse mammary tumor virus (MMTV)-long terminal repeat (LTR) develop mammary tumors. Analyses of the tumors show overexpression of β -catenin as well as cyclin D1. Thus, antagonism of GSK3 β activity is oncogenic in the mammary epithelium; mutation or pharmacologic down-regulation of GSK3 β could promote mammary tumors.

Materials and Methods

Cloning of mouse glycogen synthase kinase 3 β and kinase-inactive murine glycogen synthase kinase 3 β . Based on the sequence of human GSK3 β , we synthesized a pair of primers and amplified a portion of the mGSK3 β cDNA. A randomly primed murine spleen Lambda ZAP II cDNA library (Stratagene, La Jolla, CA) was screened with the radiolabeled murine PCR product and a single clone was isolated that contained a full-length GSK3 β open reading frame (ORF) based on bidirectional sequencing (DNA/Protein Core Facility, Boston University School of Medicine, Boston, MA). Double-stranded site-directed mutagenesis was done on the mGSK3 β sequence to introduce a mutation into the ATP-binding site, changing KKV at amino acids 85 to 87 to MII with the expectation of creating an inactive enzyme, mGSK3 β -KI. This created an additional *Bcl*II site, so PCR was used to amplify the fragment containing the mutation and distinguish it by *Bcl*II digestion, and it was also confirmed by sequencing. For *in vitro* experiments, the KI-mGSK3 β cDNA was subcloned into pcDNA3.0 (Invitrogen, Carlsbad, CA). Using PCR, a hemagglutinin (HA) tag was added 5' to the KI-mGSK3 β coding sequence in this vector. The sense oligonucleotide primer contained HA tag and the KI-mGSK3 β sequence (5'-GGGGTACCACCACCATGGCC-TACCCATACGACGTACCAGACTACGCATCGGGGCGACCGAGAACCACC-3') and an antisense oligonucleotide was from the end of the ORF (5'-GGTCTAGAGCTCCTGGGGGCTGTTCAGG-3'). Parallel experiments to those with the HA-KI-mGSK3 β were carried out with a myc-tagged kinase-inactive mutant of rat GSK3 β (rGSK3 β), myc-rGSK3 β (K85R).

Cell culture. C57MG normal mouse mammary epithelial cells were grown in DMEM supplemented with 10% fetal bovine serum, 4 mmol/L glutamine, 50 units/mL penicillin, and 50 mg/mL streptomycin (Cellgro, Mediatech, Inc., Herndon, VA) in a 5% CO $_2$ incubator at 37°C. Transfections were done using LipofectAMINE 2000 (Invitrogen) or the Amaxa nucleofection system according to the manufacturer's instructions. The total amount of transfected DNA was kept constant by adding plasmid vector DNA when necessary. For small interfering RNA (siRNA) experiments, cells were transfected with SMARTpool mGSK3 β siRNA or siCONTROL (Dharmacon, Chicago, IL). Alternatively, cells were treated with the GSK3 inhibitors SB216763 (Sigma, St. Louis, MO) or TDZD-8 (Calbiochem, San Diego, CA).

Western blot analyses. Protein extracts were prepared by homogenizing frozen tumors or mammary gland specimens in lysis buffer as described (34). Primary antibodies were the following monoclonal antibodies: anti- β -catenin (BD, Lexington, KY), anti- β -actin (Sigma), anti-cyclin D1

(Calbiochem), anti- α -tubulin (Sigma), anti-HA.11 (Covance/Babco, Richmond, CA), anti-*c-myc* (Roche, Indianapolis, IN), and anti-SP1 (BD). For quantitative analysis of each band, integrated pixel density minus background density was determined using a Fluor-S MultiImager and analysis was done using Quantity One software (Bio-Rad, Hercules, CA).

For nuclear and cytoplasmic separations, cells were washed, harvested with ice-cold PBS, and centrifuged at $960 \times g$ for 5 minutes at 4°C. The pellet was suspended in 2 volumes of ice-cold, low-salt buffer [10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl $_2$, 10 mmol/L KCl, 0.05% NP40, 0.5 mmol/L DTT] supplemented with protease inhibitor cocktail (Sigma) and incubated on ice for 30 minutes. Following 15 minutes of centrifugation at $10,600 \times g$, the supernatants were frozen as cytoplasmic extracts. Nuclei were extracted with 2 volumes of ice-cold, high-salt buffer [20 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl $_2$, 0.42 mol/L NaCl, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 25% (v/v) glycerol] and incubated at 4°C for 40 minutes. Nuclear extracts were cleared by centrifugation at $20,800 \times g$ for 15 minutes.

Immunofluorescence microscopy. C57MG cells were transfected with HA-KI-GSK3 β using the Amaxa nucleofection system and plated on glass coverslips. Twenty-four hours later, the transfected cells were transferred into 1% fetal bovine serum in DMEM, starved overnight, and then stained. Cells were washed thrice with cold PBS and then fixed and permeabilized with 4% paraformaldehyde and 0.5% Triton X-100 for 10 minutes, blocked with 3% bovine serum albumin for 30 minutes, and subsequently incubated at 4°C with primary anti- β -catenin antibody and secondary FITC-conjugated anti-mouse IgG (Sigma) for 60 minutes each. For nuclear staining, we used Hoechst dye by adding a 1:100 dilution of 100 μ g/mL stock to the medium of the cells 20 minutes before the beginning of the experiment. Pictures were taken in a fluorescence microscope (Nikon, Japan) fitted with a digital camera (Diagnostic Instruments, Sterling Heights, MI). The software used was Spot Advance (Diagnostic Instruments).

β -catenin protein stability. C57MG cells (0.5×10^6) were transiently transfected with different amounts of the HA-KI-GSK3 β construct. After 24 hours, the cells were starved in 1% fetal bovine serum and 16 hours later were treated with 50 μ g/mL cycloheximide to block *de novo* protein synthesis. Samples were taken at the beginning of the experiments and at 2-hour intervals. At each time point, the cells were washed in cold PBS and pelleted, proteins were extracted, and Western blotting was done for β -catenin.

Quantitative real-time PCR and semiquantitative reverse transcription-PCR. Reactions (25 μ L) were prepared by mixing 12.5 μ L of Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA), 5 ng of the relevant cDNA, and 1.25 μ L of an Assay-on-Demand gene expression product for cyclin D1 (CCND1) or β -glucuronidase (GUSB) as an endogenous control. Quantitative real-time PCR (qPCR) was done in an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The initial step was for 10 minutes at 95°C and then 40 cycles of denaturation at 95°C for 15 seconds and annealing/extending at 60°C for 1 minute. Background signal was eliminated and Ct values were determined using the SDS version 1.1 analysis software (Applied Biosystems). Standard curves for CCND1 and GUSB were done to confirm that the sample Ct values were within the range. For reverse transcription-PCR (RT-PCR), total RNA (1 μ g) was reverse transcribed using the ProSTAR First-Strand RT-PCR kit (Stratagene). PCR with specific primers for cyclin D1 (sense 5'-CCCTCCGTATCTTACTTCAA-3' and antisense 5'-GATGGTCTGCTTGTCTCAT-3') was done in a thermal cycler (MJ Research, Watertown, MA) by denaturing at 95°C for 3 minutes and then 30 cycles of denaturing at 95°C for 30 seconds, annealing at 53°C for 30 seconds, and extending at 72°C for 30 seconds.

Transgenic animals. The KI-mGSK3 β cDNA was subcloned into a vector in which the MMTV-LTR directs expression to the mammary epithelium, with *ras* 5' untranslated sequences provided upstream of the cDNA and a SV40 intron and polyadenylation signal downstream (35). Plasmid sequences were removed by restriction digestion at the *Sa*I and *Spe*I sites, and the excised transgene construct was gel purified and microinjected into pronuclei of fertilized one-cell zygotes of FVB/N mice in the Transgenic Core Laboratory at Boston University School of Medicine. Three independent transgenic lines were obtained, and female transgenic mice were continuously bred to induce transgene expression through

activation of the hormone-dependent MMTV-LTR. Mice were monitored weekly for the appearance of tumors. Mice ($n = 117$) were sacrificed and tissues were collected for histopathological analysis, cell culture, and RNA and/or protein analyses. To assess expression levels of the transgene in the mouse organs, tissues were collected from 6- to 8-week-old females that were pregnant and from females that developed mammary tumors.

Expression analyses. For expression analysis, total RNA was extracted from mouse tissues. After DNase treatment (Roche), RNA was reextracted and ethanol precipitated. RNA (5–10 μ g) was then reverse transcribed using the ProSTAR First-Strand RT-PCR kit. PCR was done with sense GSK3 β (CGAGACACACCTGCACTCTT) and SV40 primers described above, for 35 cycles, to detect the transgene. Both spliced and unspliced transgene mRNA could be amplified (614 and 547 bp), as there is a splice donor and acceptor in the amplified region of the SV40 poly(A) tail. The quality of first-strand synthesis was verified with *HPRT* amplification (36).

Histology. On necropsy, tumors and organs were removed and immediately fixed in Optimal Fix (American Histology Reagent Co., Inc., Lodi, CA). The tissues were processed, embedded in paraffin, and sectioned at 7 μ m. The sections were mounted on glass slides and stained with H&E using routine laboratory procedures in the Transgenic Core Pathology Laboratory at the University of California-Davis (Davis, CA). Immunohistochemistry for cytokeratins, smooth muscle actin, hair keratin, and estrogen and progesterone receptors were done as described previously (37). Images were captured with $\times 10$, $\times 20$, and $\times 40$ objectives using a Carl Zeiss (Thornwood, NY) Axiocam camera and processed using Adobe Photoshop (Adobe Systems, Inc., San Jose, CA) software. Sections were compared with other specimens in the extensive mouse mammary tumor database.³

Results

Expression of myc-rGSK3 β (K85R) up-regulates β -catenin in mammary epithelial cells. As a first step to determine whether dysregulation of GSK3 β could play a role in mammary carcinogenesis, we manipulated its levels in cells *in vitro* and assessed the expression of β -catenin as an indicator of Wnt signaling. Initial experiments were carried out with a previously described rat construct (4). C57MG cells, a nonmalignant cell line derived from murine breast epithelial cells, were transiently transfected by nucleofection with increasing amounts of myc-tagged kinase-inactive mutant form of rGSK3 β , myc-rGSK3 β (K85R), construct. pEGFP-C1 was cotransfected; the transfection efficiency using this method was 75% (data not shown). After 48 hours, protein extracts were analyzed for β -catenin expression. β -catenin protein levels were up-regulated with increasing expression of the myc-rGSK3 β (K85R) (Fig. 1A), consistent with increasing activation of the Wnt pathway.

myc-rGSK3 β (K85R) stabilizes endogenous β -catenin protein. In the absence of Wnt signals, β -catenin is incorporated into a cytoplasmic complex, including GSK3 β , which targets it for degradation. To validate that the changes in β -catenin expression are due to ability of the myc-rGSK3 β (K85R) to stabilize β -catenin protein, we studied the half-life of β -catenin. C57MG cells were transiently transfected with increasing amounts of the myc-rGSK3 β (K85R) construct. Forty-eight hours after transfection, the cells were treated with cycloheximide to block *de novo* synthesis of β -catenin, and periodically, cells were harvested and proteins were subjected to immunoblotting to assess β -catenin stability. The half-life of β -catenin was increased, being 1.5, 5.4, 26, and 11 hours in the cell lines expressing 0, 2, 4, or 6 μ g of the myc-rGSK3 β (K85R), respectively (Fig. 1B).

β -catenin is localized to the nucleus in mammary cells expressing myc-rGSK3 β (K85R). When Wnt signaling is activated, free β -catenin is translocated into the nucleus to stimulate

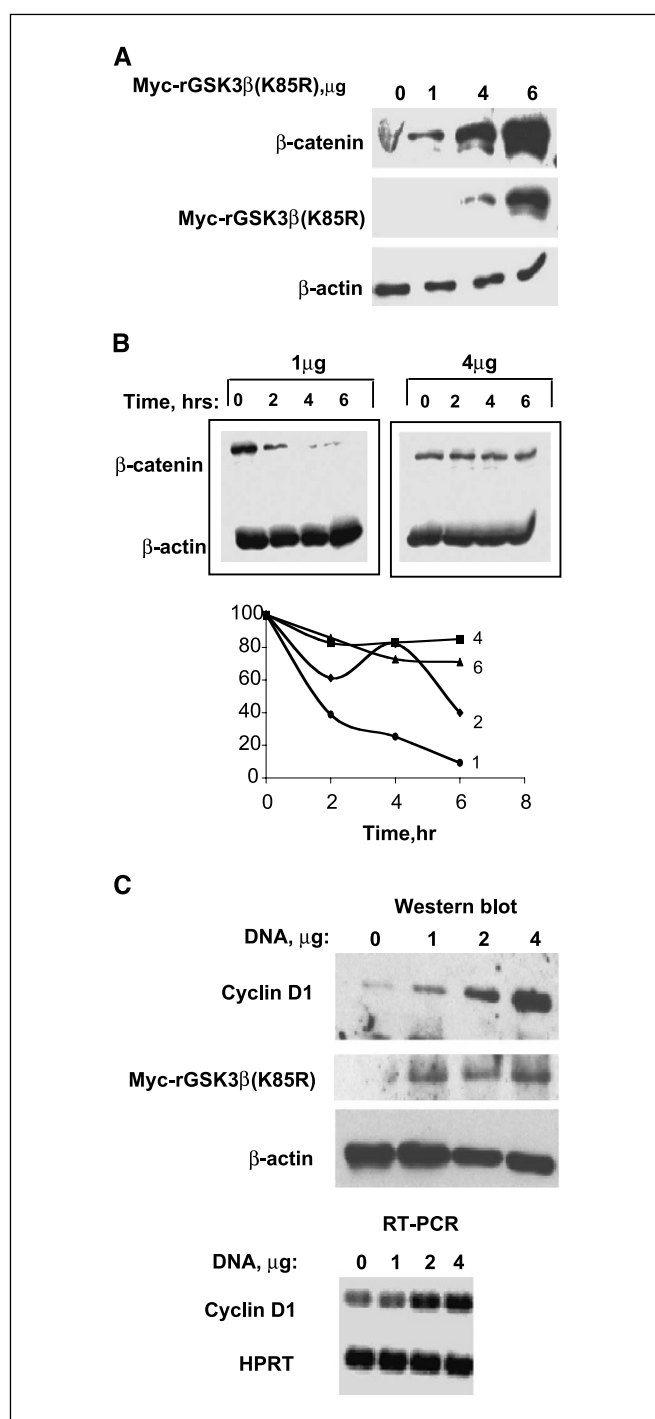


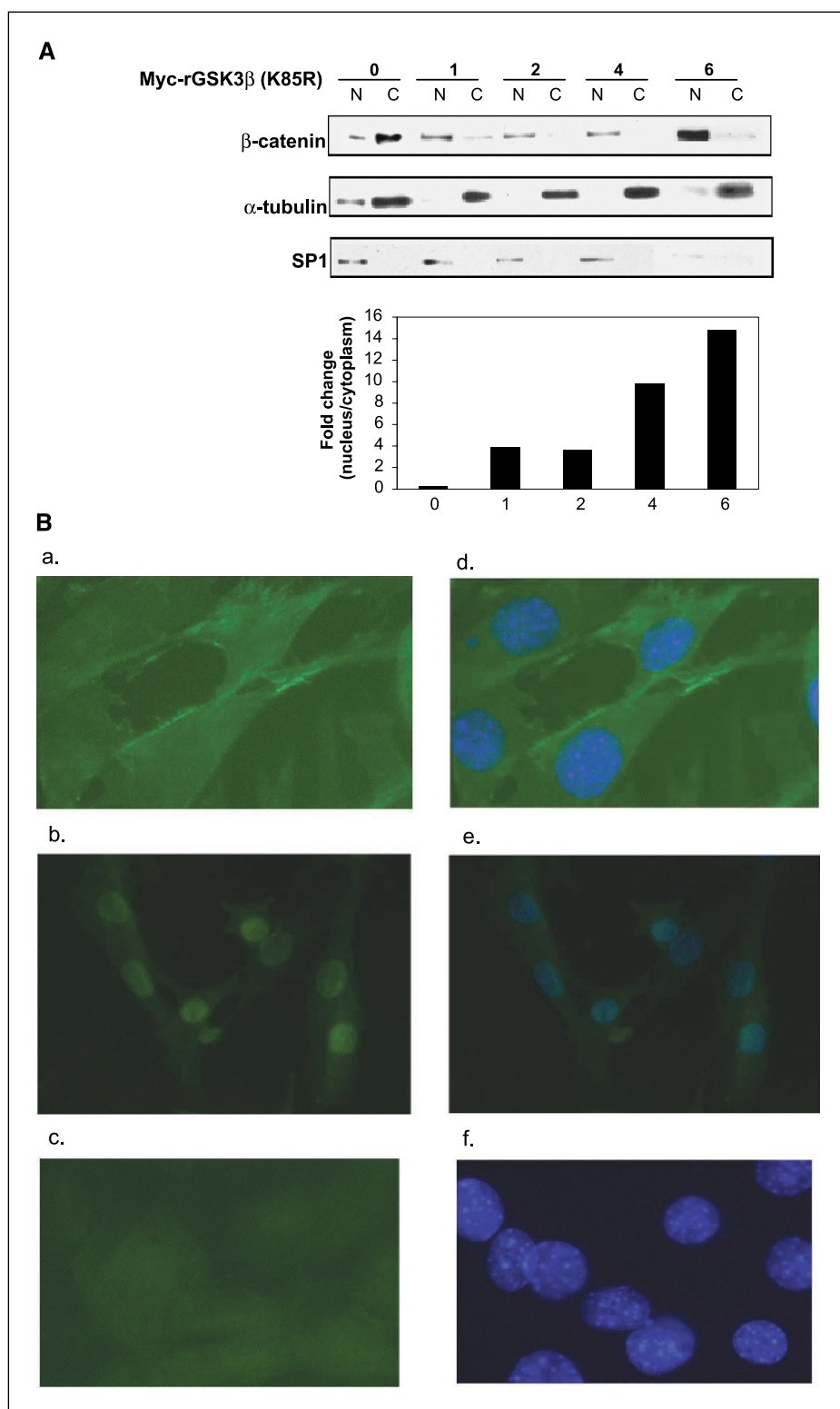
Figure 1. Expression of myc-rGSK3 β (K85R) up-regulates β -catenin expression in mammary epithelial cells. **A**, expression of myc-rGSK3 β (K85R) up-regulates β -catenin expression in C57MG cells. Increasing amounts of myc-rGSK3 β (K85R) plasmid (in μ g) were transiently transfected into C57MG cells. Protein (5 μ g) extracted from the cell was subjected to immunoblotting for β -catenin; myc-rGSK3 β (K85R) was detected using an anti-myc antibody; β -actin was used as a loading control. **B**, transfection with myc-rGSK3 β (K85R) plasmid (1–6 μ g) increases the half-life of β -catenin protein. Cells were treated with 50 μ g/mL cycloheximide 48 hours after transfection. *Top*, two representative blots; *bottom*, relative percentage of protein compared with time 0, normalized to β -actin. **C**, cyclin D1 expression is up-regulated in C57MG cells overexpressing myc-rGSK3 β (K85R). *Top*, protein (10 μ g) extracted from the cells expressing increasing amounts of myc-rGSK3 β (K85R) plasmid (0–4 μ g) was subjected to immunoblotting for cyclin D1 and for the myc tag; β -actin was used as a loading control. *Bottom*, RNA was prepared from the same cells and subjected to RT-PCR for cyclin D1; hypoxanthine phosphoribosyltransferase (*HPRT*) was used as a control.

³ <http://tgmouse.compmed.ucdavis.edu>.

transcription of proto-oncogenes, such as *cyclin D1* and *c-myc*. To investigate if myc-rGSK3 β (K85R) can influence the translocation of β -catenin from the cytoplasm to the nucleus, cytoplasmic and nuclear extracts were prepared from the transfected C57MG cells. In the control cells transfected with the empty vector alone, the levels of β -catenin protein in the cytoplasm were higher than in the

nucleus. In contrast, in the presence of increasing amounts of myc-rGSK3 β (K85R), the levels of β -catenin in the nucleus were significantly higher than in the cytoplasm (Fig. 2A). Quantification showed a ratio of nuclear-to-cytoplasmic expression of 0.3, 1.9, 3.9, 3.5, 9.8, and 14.8 in cells transfected with 0, 1, 2, 4, or 6 μ g of the myc-rGSK3 β (K85R), respectively (Fig. 2A).

Figure 2. β -catenin is localized to the nucleus in mammary cells overexpressing HA-KI-mGSK3 β and myc-rGSK3 β (K85R). **A**, 48 hours after transient transfection of increasing amounts of myc-rGSK3 β (K85R) plasmid (0-6 μ g), nuclear (N) and cytoplasmic (C) extracts were prepared from C57MG cells. Immunoblotting for β -catenin was done; α -tubulin was used as a cytoplasmic control and SP1 as a nuclear control. **Bottom**, ratio of the amount of protein in the nucleus to that in the cytoplasm. **B**, cells were subjected to immunofluorescence using a monoclonal antibody against β -catenin and the nuclei were stained with Hoechst dye: (a) empty vector (FITC alone) and (d) merged with the Hoechst staining, (b) 4 μ g HA-KI-mGSK3 β and (e) merged with Hoechst, and (c) FITC secondary antibody control and (f) merged with Hoechst.



A murine kinase-inactive form of glycogen synthase kinase 3 β . We cloned the full-length ORF of mGSK3 β from a phage library; its sequence was identical to the sequence in Genbank (gi:7025914). To create an inactive enzyme that might function as a dominant negative for signaling in the Wnt pathway, we mutated the ATP-binding site, similar to what was done for the human GSK3 β (5). *In vitro* translated wild-type (WT) and mutant GSK3 β constructs were subjected to a kinase assay using a GSK3 β substrate peptide derived from cyclic AMP-responsive element-binding protein (38). The activity of the mutant was <20% of that of the WT enzyme, consistent with its design as a kinase-inactive mutant (data not shown). Moreover, to further confirm the ability of the KI-mGSK3 β construct to act as a dominant negative, we tested its ability to produce axis duplication in *Xenopus* embryos as has been reported for the mutated inactive human GSK3 β (5). RNA for KI-mGSK3 β was transcribed *in vitro* and injected ventroequatorially into *Xenopus* embryos, and these embryos developed ectopic axes consistent with activation of the Wnt pathway (data not shown).

Wnt pathway activation in cells *in vitro* by HA-KI-mGSK3 β . We confirmed that HA-KI-mGSK3 β acts in the same fashion as myc-rGSK3 β (K85R). Steady-state elevation of β -catenin expression was detected in C57MG cells transiently transfected with increasing expression of HA-KI-mGSK3 β (Fig. 3A). The half-life of β -catenin in the transfected cells was as long as 26.6 hours (4 μ g plasmid) compared with only 2.2 hours in untransfected cells (Fig. 3B). Immunofluorescence was used to confirm nuclear translocation of the up-regulated β -catenin. C57MG cells transfected with 4 μ g of the HA-KI-mGSK3 β construct or empty vector were fixed and stained with a primary monoclonal antibody against β -catenin and a secondary antibody conjugated with FITC along with Hoechst dye to identify the nuclei. In the control cells (Fig. 2B, a and d), most of the β -catenin is located in the cytoplasm and in the plasma membrane. In contrast, the cells transfected with the HA-KI-mGSK3 β plasmid exhibited strong nuclear staining (Fig. 2B, b and e). As a control for nonspecific fluorescence, the FITC secondary antibody was used alone on the same transiently transfected cells (Fig. 2B, c and f). Thus, the mutant HA-KI-mGSK3 β acts similarly to the myc-rGSK3 β (K85R) in promoting β -catenin stabilization and nuclear translocation, consistent with canonical Wnt pathway activation.

Inhibition of murine glycogen synthase kinase 3 β using small interfering RNA or pharmacologic inhibitors up-regulates β -catenin expression. We compared our *in vitro* results with the HA-KI-mGSK3 β construct to other methods of inhibiting GSK3 β . C57MG cells were transiently transfected by nucleofection with SMARTpool siRNA, a pool of four specific siRNAs for mGSK3 β , or siCONTROL. GSK3 β proteins levels were reduced significantly in cells transfected with the siRNAs for mGSK3 β compared with siRNA control or untransfected cells (Fig. 4A). There was an inverse correlation between the expression of GSK3 β and β -catenin, which was up-regulated in cells transfected with GSK3 β siRNA. Alternatively, C57MG cells were treated with the GSK3 inhibitors SB216763 or TDZD-8 at 20 and 5 μ mol/L, respectively. As expected, the kinase inhibitors did not alter the levels of GSK3 β but up-regulated β -catenin expression, consistent with inhibition of GSK activity (Fig. 4B and C). Thus, *in vitro*, the mutant HA-KI-mGSK3 β construct acts similarly to specific GSK3 β siRNA and pharmacologic inhibitors.

Cyclin D1 is up-regulated in mammary cell lines transfected with HA-KI-mGSK3 β . When β -catenin translocates into the

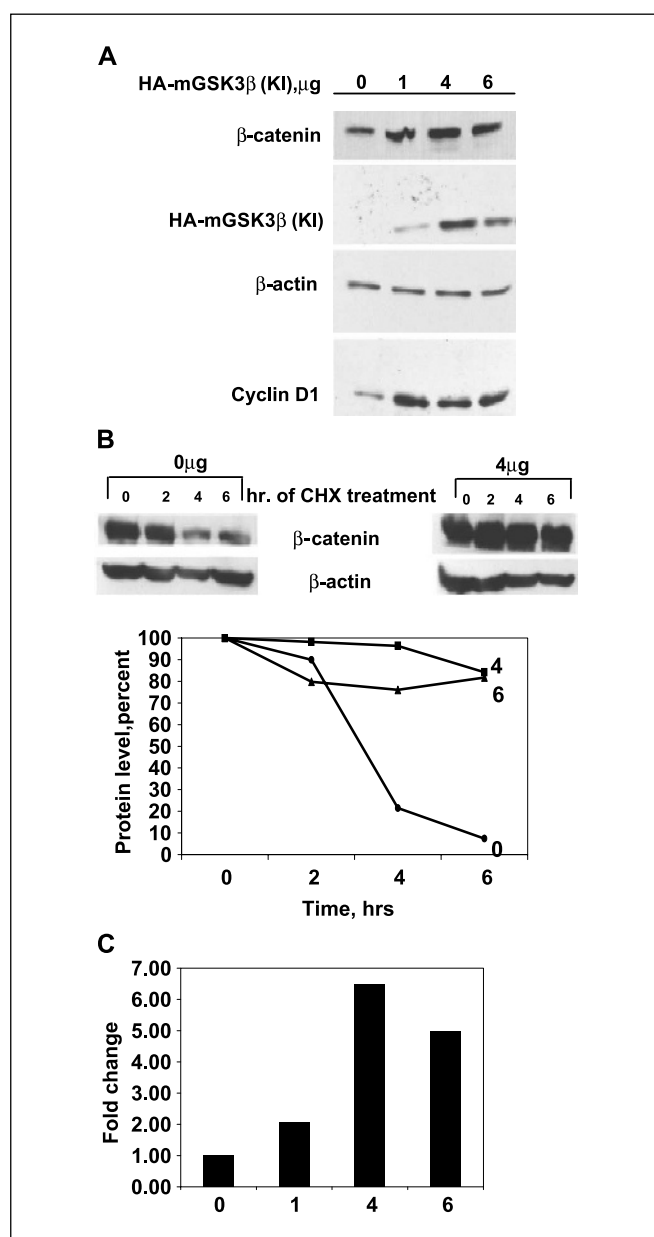


Figure 3. Overexpression of HA-KI-mGSK3 β up-regulates β -catenin and cyclin D1 expression in mammary cells. **A**, to confirm that the HA-tagged mGSK3 β also regulates β -catenin, increasing amounts (0–6 μ g) were transiently transfected into C57MG cells and expression of β -catenin and cyclin D1 was determined. **B**, half-life of β -catenin protein was measured. **C**, amount of cyclin D1 mRNA was quantitated using real-time PCR and compared with GUSB as a control; the ratios of the two are plotted based on Ct values. HA-KI-GSK3 β plasmid (0, 1, 4, and 6 μ g) was transfected.

nucleus during canonical Wnt signaling, it binds the factors of the TCF/LEF family and dramatically increases their transcriptional activity, stimulating the expression of proto-oncogenes, such as *cyclin D1* (13, 14). In a preliminary experiment, we used the TOPFLASH/FOPFLASH TCF/LEF luciferase reporter system (39) and showed that cotransfection of the reporter along with HA-KI-mGSK3 β resulted in a 10-fold increase in luciferase activity compared with controls (data not shown). To show this for an endogenous biologically relevant gene, we measured levels of cyclin D1 in transiently transfected C57MG cells. Semiquantitative PCR with specific primers for cyclin D1 suggested that there was

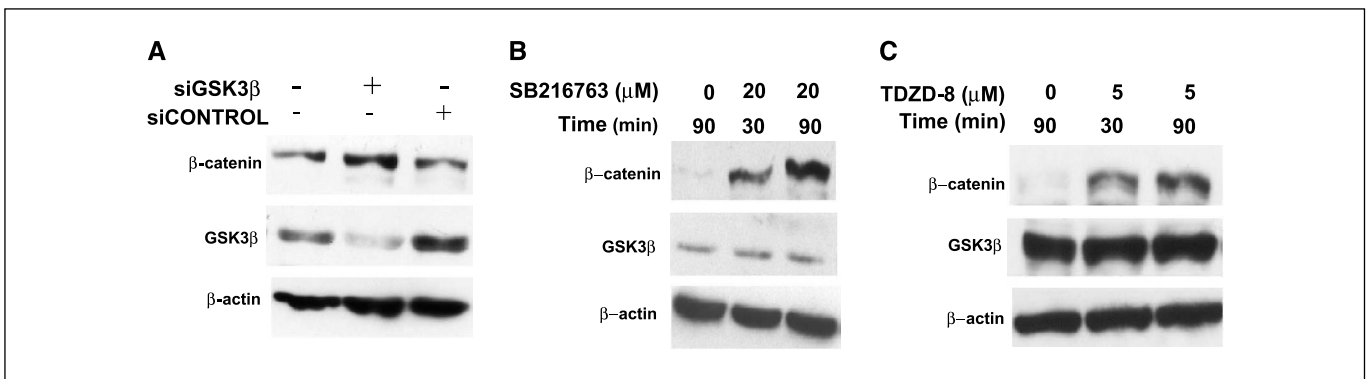


Figure 4. Inhibition of mGSK3 β using siRNA or pharmacologic inhibitors up-regulates β -catenin expression. **A**, inhibition of GSK3 β by siRNA up-regulates β -catenin. C57MG cells were transiently transfected with siRNAs for mGSK3 β or siCONTROL. Twenty-four hours later, protein (10 μ g) extracted from the cells was subjected to immunoblotting for GSK3 β and β -catenin; β -actin was used as a loading control. **B** and **C**, pharmacologic inhibitors of GSK3 β up-regulate β -catenin expression. C57MG cells were treated with DMSO vehicle control (0), SB216763 (20 μ mol/L), or TDZD-8 (5 μ mol/L) for 30 or 90 minutes. Protein extracted from the cells was subjected to immunoblotting for GSK3 β and β -catenin; β -actin was used as a loading control.

an increased amount of cyclin D1 mRNA in cells expressing the HA-KI-mGSK3 β (data not shown). These results were confirmed using qPCR, where an increase in cyclin D1 mRNA of up to \sim 7-fold was seen (Fig. 3C). Consistent with these results, the levels of cyclin D1 protein were higher in cells expressing HA-KI-mGSK3 β compared with vector-transfected cells (Fig. 3A, bottom). Similar results were obtained with the myc-rGSK3 β (K85R) construct (Fig. 1C), suggesting that kinase-inactive forms of GSK3 β are able to promote complete and functional Wnt signaling, including up-regulation of target genes.

Transgenic expression of kinase-inactive murine glycogen synthase kinase 3 β in the mouse mammary gland. The KI-mGSK3 β construct was subcloned into a MMTV-LTR vector (40), designed for hormone-dependent transgene expression in the adult mammary gland, and microinjected into fertilized FVB/N mouse oocytes. Three founders were identified by Southern blotting; their offspring had similar expression and phenotypes, so the data were pooled for all three lines. To verify the expression of the transgene mRNA, a transgene-specific RT-PCR assay was employed. During pregnancy, which activates transgene expression, the mammary glands and other epithelial tissues, including kidney, small intestine, salivary gland, and spleen, expressed transgene-specific transcripts (data not shown), a pattern of expression has been seen with other MMTV transgenes (35). To determine whether the transgene protein is functional *in vivo* as a Wnt pathway activator, we assayed for expression of GSK3 β and β -catenin protein in the mammary gland. Although the untagged kinase-inactive protein could not be distinguished from the WT kinase by immunoblot, we found an increase in total GSK3 β protein in the mammary gland of the transgenic compared with WT mice along with a significant increase in β -catenin protein, consistent with Wnt pathway activation by the transgene (Fig. 5A).

Mammary tumors in MMTV-KI-mGSK3 β transgenic mice. MMTV-KI-mGSK3 β mice develop and breed normally. To promote transgene expression from the hormone-dependent MMTV-LTR, female mice were continuously bred and pups were removed after 7 days of lactation. A cohort of 117 transgenic female mice derived from the three independent transgenic lines was observed for 2 years. Sixty-two percent of the mice developed mammary tumors at a median age of 22 months, with no significant difference in incidence among the lines. Although they

occur long after pregnancy, the tumors continue to express the transgene (Fig. 5B). The pooled mammary tumor incidence is illustrated in a Kaplan-Meier plot (Fig. 5C). The tumors and other mammary glands and organs were harvested for histologic and molecular analyses.

Detailed histopathological analyses were done on 54 of the female transgenic mice that had evidence of mammary tumors (Table 1; Fig. 6). Most of the tumors were adenocarcinomas (Fig. 6A), including variants such as papillary carcinomas (Fig. 6B), and the tumors were frequently associated with invasive growth (Fig. 6C). The most common histologic subtypes were pilar (squamous) tumors ($n = 13$), papillary tumors ($n = 12$) typically with micropapillary components, glandular tumors ($n = 8$), and myoepithelial tumors ($n = 8$ spindle cell tumors and $n = 1$ adenomyoepithelioma). The tumors tended to be stroma rich, to contain inflammatory infiltrates, and to keratinize. Immunohistochemistry was negative for estrogen and progesterone receptors, except in the spindle cell tumors, which had perinuclear estrogen receptor staining and were also positive for smooth muscle actin (data not shown). Immunofluorescence for cytokeratin 1 (Fig. 6E), cytokeratin 5, cytokeratin 6, and hair keratin (not shown) confirmed transdifferentiation into epidermal and pilar structures. Other mice had hyperplastic and dysplastic mammary lesions without tumors. Twenty-one mice had other malignancies, including lymphomas ($n = 8$), leukemias ($n = 6$), bronchoalveolar lung tumors ($n = 5$), and hepatoma ($n = 2$). The incidence of nonmammary neoplasms was very similar to that reported for WT mice of the same FVB/N strain (41) and thus most likely does not result from an effect of the transgene.

Transgenic expression of kinase-inactive murine glycogen synthase kinase 3 β up-regulates β -catenin expression and cyclin D1 *in vivo*. To confirm that tumorigenesis in the MMTV-KI-mGSK3 β was occurring in association with activation of Wnt signaling, we assayed the expression levels of β -catenin in mammary glands and tumor tissues from the transgenic mice. β catenin protein was up-regulated in the tumor samples in six of seven transgenic mice (Fig. 7A, quantification in Fig. 7B). In these tumors, cyclin D1 was also up-regulated (Fig. 7A).

Using qPCR, we compared expression of β -catenin and cyclin D1 in the mammary glands of WT female FVB/N mice and MMTV-KI-mGSK3 β transgenics. We found that the presence of

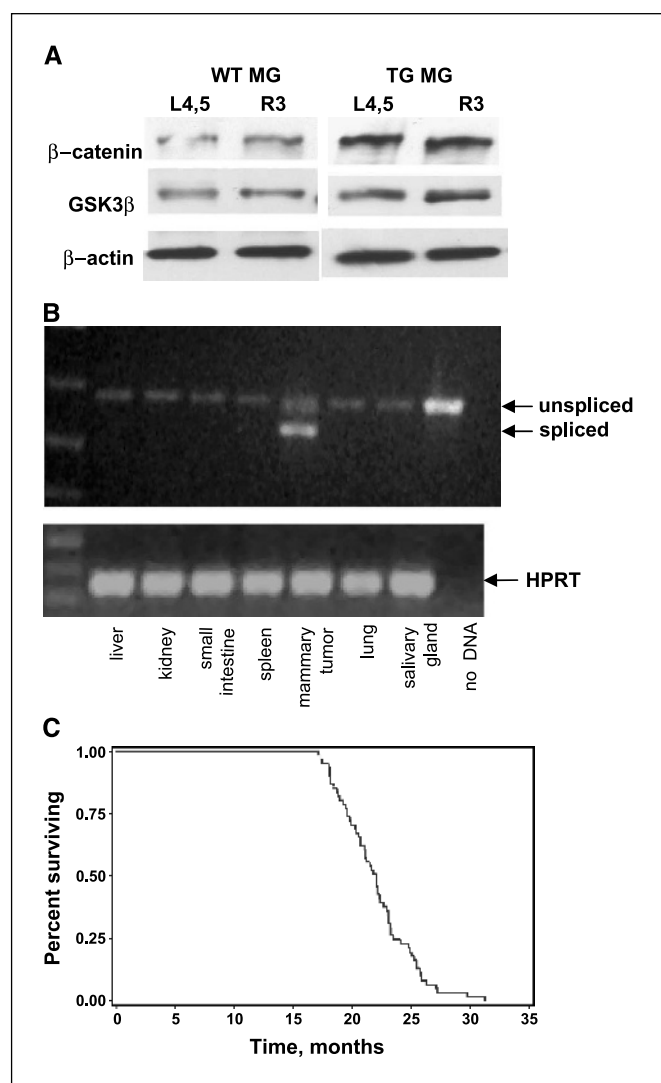


Figure 5. Transgene expression of MMTV-KI-mGSK3 β *in vivo*. **A**, expression of GSK3 β and β -catenin protein in the mammary glands. Protein (15 μ g) extracted from mammary glands (MG) of transgenic (TG) or WT female mice was subjected to immunoblotting for GSK3 β and β -catenin proteins; β -actin was used as a loading control. **B**, RT-PCR analysis of MMTV-KI-mGSK3 β transgene expression in a mouse with a mammary tumor. Total RNA (10 μ g) derived from the indicated organs was subjected to RT-PCR with a GSK3 β forward primer and a SV40 reverse primer that encompass a 67-bp splicing region in the SV40 poly(A) tail, yielding a 614-bp unspliced mRNA band or the mature 547-bp mRNA; *HPRT* amplification confirmed the integrity of the reverse transcription reaction. **C**, Kaplan-Meier plot of incidence of mammary tumors in three lines of MMTV-KI-GSK3 β transgenic female mice, continuously mated to induce transgene expression.

the transgene resulted in a detectable increase in cyclin D1 mRNA (Fig. 7C, *black columns*) but not β -catenin mRNA (Fig. 7C, *gray columns*) in the premalignant mammary gland as well as in malignant mammary gland.

Discussion

We have engineered a murine KI-GSK3 β by altering three residues (85-87) in the ATP-binding site of mGSK3 β , similar to what was done for the human GSK3 β (5). We compared it to a previously described GSK3 β (K85R) construct that has been shown to activate Wnt signaling in rat-1 fibroblasts and PC12 cells (42).

The construct had minimal kinase activity against a peptide substrate, consistent with its design as a kinase-inactive mutant. To confirm its ability to act as a dominant negative and stimulate Wnt signaling in the mammary epithelium, we tested the construct both *in vitro* and *in vivo*. *In vitro*, we used C57MG cells, a nonmalignant cell line derived from murine breast epithelial cells. These cells are responsive to Wnt signaling; expression of Wnt in C57MG cells causes morphologic transformation and apparent loss of contact inhibition of cell growth (43). We studied the expression, half life, and subcellular localization of β -catenin and other target proteins and genes in C57MG cells transfected with increasing amounts of HA-KI-mGSK3 β . We found that KI-mGSK3 β stabilizes β -catenin expression and promotes its translocation to the nucleus. We also detected morphologic differences in C57MG cells transfected with HA-KI-mGSK3 β ; the cells were less spread out and there was a reduction in membrane staining of β -catenin probably because of diminished interaction between β -catenin and E-cadherin in those cells. The previously reported myc-rGSK3 β (K85R) was used in the same assays and gave similar results (4). We compared these findings to other known methods for inhibiting GSK3 β , such as siRNA and pharmacologic inhibitors, and obtained similar results. The key role of GSK3 β as a negative regulator of Wnt signaling has also been shown using siRNA in mouse P19 cells (44).

In vitro, the HA-KI-mGSK3 β promoted Wnt-dependent transcription based on its ability to activate a TCF/LEF-dependent reporter and to up-regulate cyclin D1 mRNA and protein. Cyclin D1 overexpression has been found in 50% of patients with breast cancer, but only 15% to 20% of these cases show gene amplification of cyclin D1 (45-47). Other mechanisms, such as up-regulation of gene transcription and translation, also play roles in overexpression of cyclin D1 in breast cancer; in fact, GSK3 β has been shown to directly regulate cyclin D1 turnover *in vitro* (48). β -catenin transactivation is correlated significantly with cyclin D1 overexpression in both 8 breast cancer cell lines and 123 primary breast cancer specimens (27).

In vivo, KI-mGSK3 β caused dorsal axis duplication in *Xenopus* embryos as shown previously for inactive *Xenopus*, rat, and human GSK3 β (4-6). Based on these promising results, we then established transgenic mice overexpressing KI-mGSK3 β under the control of the MMTV-LTR. Other investigators have developed transgenic models using the WT enzyme to study its role in phosphorylation of tau in the brain (49, 50), regulation of glucose

Table 1. Histologic classification of mammary tumors in KI-GSK3 β mice

	<i>n</i> (%)
Mammary tumor histology	
Adenocarcinoma	32 (59.3)
Spindle cell	8 (14.8)
Squamous	13 (24)
Adenomyoepithelial	1 (1.9)
Adenocarcinoma subtypes	
Papillary adenocarcinoma	12 (37.5)
Glandular	8 (25)
Microacinar (Dunn type A)	4 (12.5)
Tubular	3 (9.4)
Adenosquamous	5 (15.6)

metabolism in muscle (51), and its role in cardiac development (52, 53).

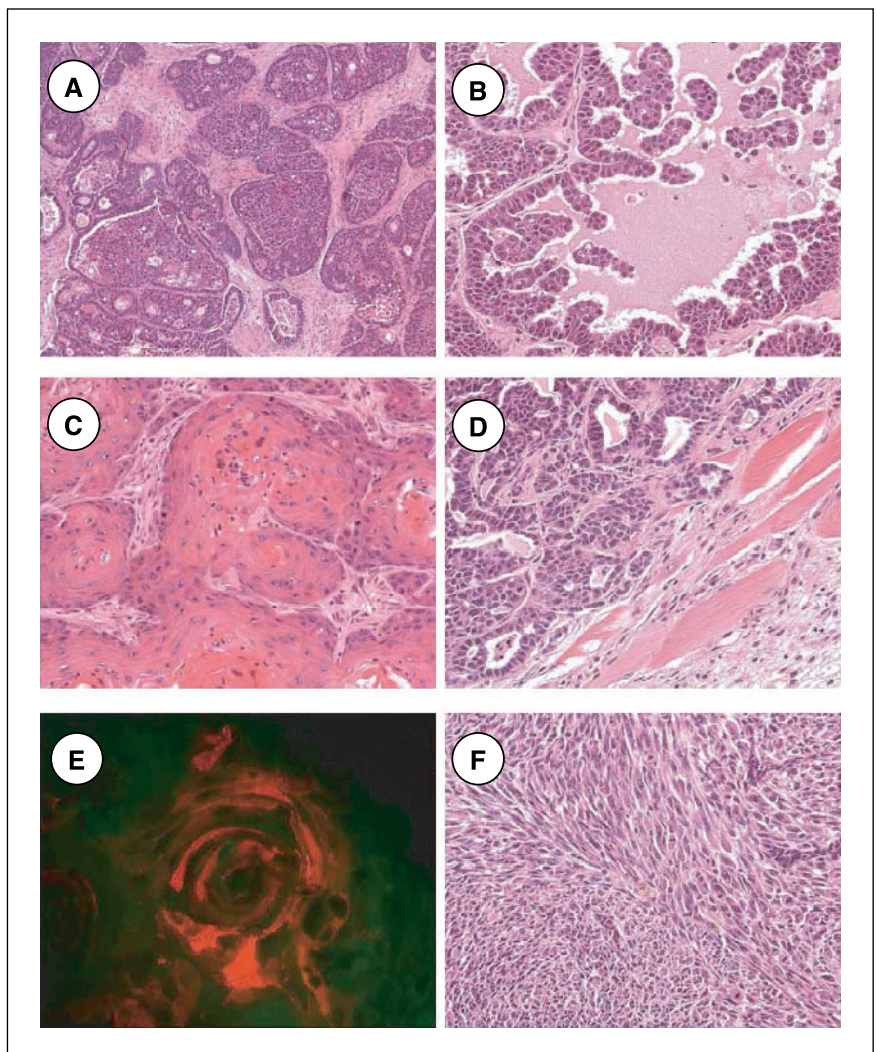
Using the MMTV promoter, KI-mGSK3 β was expressed in the adult mammary gland in response to steroid hormones and in some other epithelial tissues and T lymphocytes of pregnant mice. This pattern of expression fits with what has been seen with other MMTV transgenes (35). However, in spite of its expression in several epithelial tissues, it causes primarily mammary tumors in FVB/N mice. More than 60% of the female KI-mGSK3 β mice developed mammary tumors at a median age of 22 months. The histology and pattern of cytokeratin expression of these tumors has been compared with other murine mammary tumors and is similar to that of tumors due to other mutations in the Wnt signaling pathway, including mice with Wnt 1, Wnt 10b, and β -catenin transgenes and *APC* gene mutations (37, 54). Characteristic for these Wnt pathway-induced mammary tumors are ductular architecture, well-developed stroma, myoepithelial, acinar, or glandular differentiation, and squamous metaplasia (37). As in other mouse models with Wnt pathway activation, some KI-GSK3 β transgenic tumors showed transdifferentiation into epidermal and pilar structures accompanied by typical cytokeratin and hair keratin expression (54). This histologic determination is supported by our molecular results, as

expression of KI-mGSK3 β leads to up-regulation of β -catenin and cyclin D1.

Although tumors developing in the KI-mGSK3 β mice could theoretically result from activation of pathways other than the canonical Wnt pathway, the demonstration of up-regulation of β -catenin and the transcriptional up-regulation of the Wnt target cyclin D1 in the transgenic tumors is a strong evidence that Wnt pathway activation is a major effect of the transgene, consistent with its ability to mediate this in cells *in vitro*. Moreover, constitutive expression of Akt, in another signaling pathway that is inhibited by GSK3 β , causes delayed mammary involution but not mammary tumors when it is expressed in the mammary gland using the MMTV promoter (55, 56). This supports our contention that KI-mGSK3 β is acting through the Wnt pathway.

Thus, our experiments show that the KI-GSK3 β can promote mammary tumorigenesis. Other mutant *Wnt* genes, now well accepted to be important in human tumorigenesis, were first identified in animal models. Wnt-1 itself was cloned as a common insertion site for MMTV in murine mammary tumors (57); mutation of APC in intestinal polyps and cancers was first found as an ethylnitrosourea-induced mutation in APC^{min} mice (58). The current study suggests that GSK3 β has the capability to be a tumor suppressor, and mutations could be sought in human specimens

Figure 6. Histology of MMTV-KI-mGSK3 β transgenic mouse mammary tumors. Staining with H&E, except E. A, typical adenocarcinoma with focal squamous metaplasia; B, papillary tumor with micropapillary component; C, squamous cell carcinoma; D, invasion into chest wall; E, immunofluorescence for cytokeratin 1 of squamous nodule confirms epidermal transdifferentiation; F, spindle cell tumor.



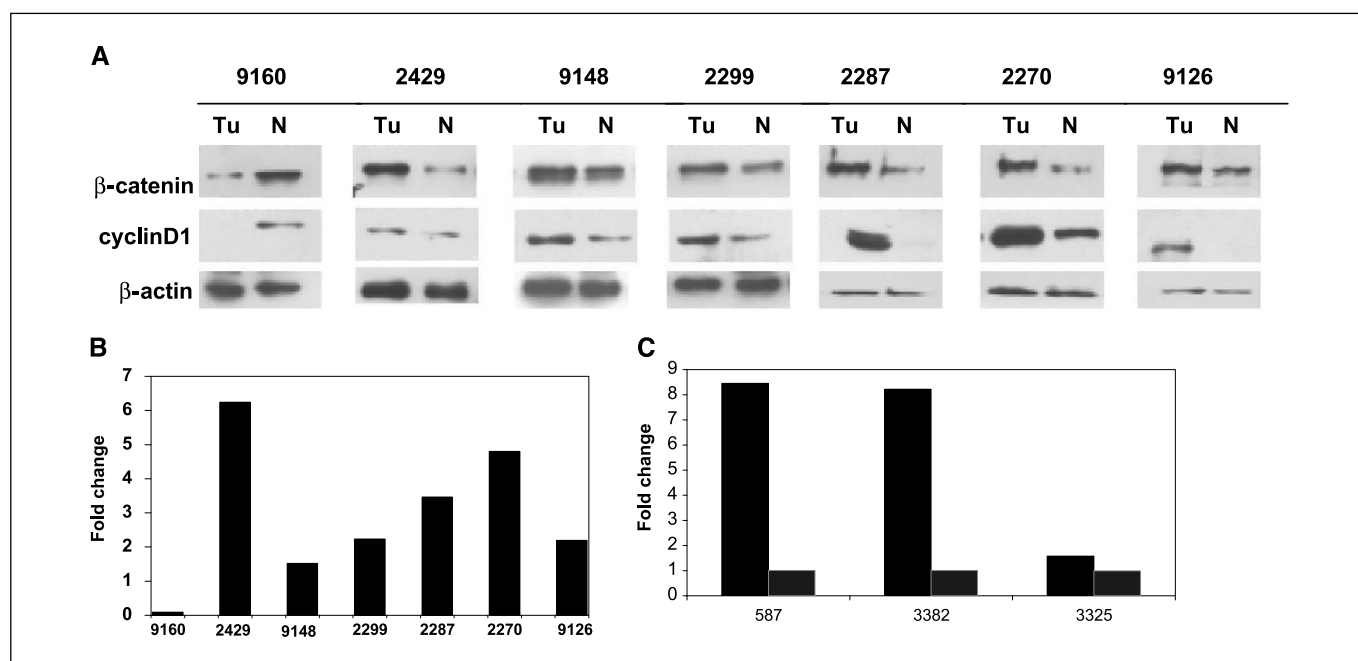


Figure 7. Expression of β -catenin and cyclin D1 protein in transgenic MMTV-KI-mGSK3 β breast tumors. **A**, protein (15 μ g) extracted from paired normal mammary glands (*N*) and mammary tumors (*Tu*) from MMTV-KI-mGSK3 β transgenic mice were subjected to immunoblotting for β -catenin and cyclin D1 and for β -actin as a loading control. **B**, ratio of β -catenin protein expression in tumor versus normal glands. **C**, mRNA levels of cyclin D1 are up-regulated in premalignant mammary gland and tumors. qPCR was used to compare the expression of cyclin D1 and β -catenin mRNA in the mammary glands of MMTV-KI-mGSK3 β transgenics to that in nontransgenic controls. mRNA was extracted from breast tumors from transgenic mice 587 and 3382 and from premalignant mammary gland from mouse 3325. Results are expressed as ratios of β -catenin mRNA (gray columns) or cyclin D1 mRNA (black columns) compared with mRNA expression in WT FVB/N female mammary gland.

from breast and other cancers. In addition, as inhibitors of GSK3 β enter clinical trials for treatment of diabetes, consideration should be given to the possibility that such drugs might up-regulate Wnt signaling and promote mammary or other tumors.

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